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LEAD IN MISSOURI STREAMS: MONITORING POLLUTION FROM MINING WITH AN ASSAY FOR ERYTHROCYTE δ-AMINOLEVULINIC ACID DEHYDRATASE (ALA-D) IN FISH BLOOD

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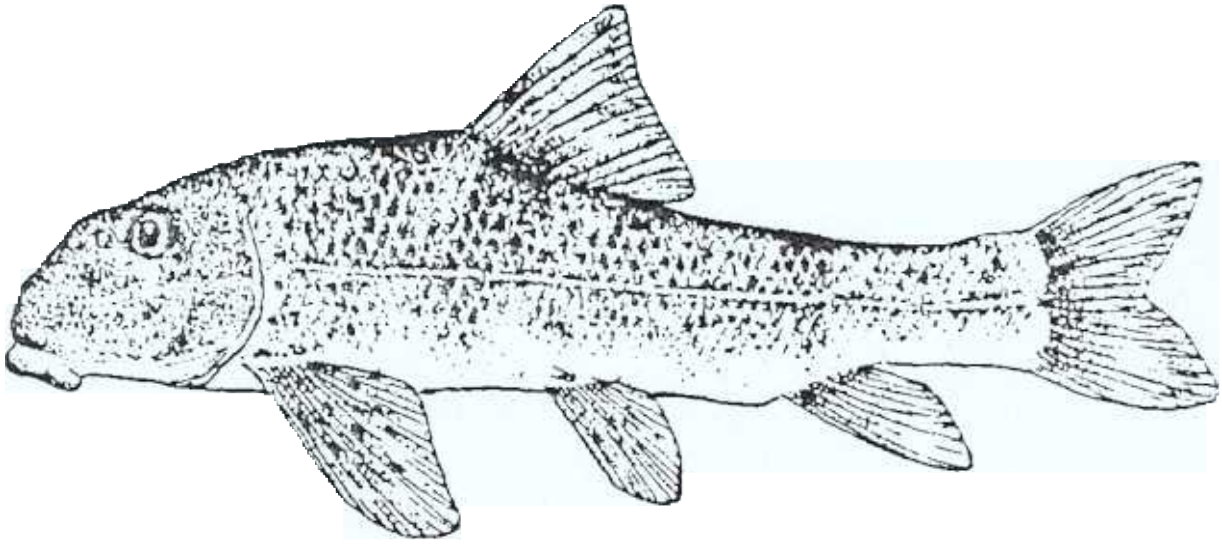

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Abstract

The activity of the erythrocyte enzyme δ-aminolevulinic acid dehydratase (ALA-D) has long been used as a biomarker of lead exposure in humans and waterfowl and, more recently, in fishes. We tested the assay for ALA-D activity in fishes from streams affected by lead in combination with other metals from lead-zinc mining and related activities. Fishes (mostly catostomids) were collected from sites affected by historic and current mining activities, and from sites thought to be unaffected by mining (reference sites). A suite of elements was measured in blood and carcass samples of individual fish and in grab samples of water and sediment from each site. ALA-D activity, total protein (TP), and hemoglobin (Hb) content also were determined in blood. Concentrations of mining-related metals (lead, zinc, and cadmium) were significantly greater ($P < 0.05$) at sites affected by historic mining activities than at reference and active mining sites. ALA-D activity, Hb, and TP accounted for 66% of blood-lead and 69% of carcass-lead variability when analyzed by multiple regression. Differences among species were minimal. ALA-D activity as a biomarker was able to successfully distinguish sites affected by bioavailable environmental lead and appeared to be unaffected by other metals except for zinc, which tended to ameliorate the inactivation of ALA-D by lead. The utility of the biomarker lies in its expedience and its ability to document bioavailability.

Introduction

Lead (Pb) was discovered in Missouri by the French explorers of the Mississippi Valley in about 1700. The exploitation of these substantial mineral resources has continued at varying levels of intensity to the present. Missouri's Pb ores also contain other extractable metals--most notably zinc (Zn), but also cadmium (Cd), copper (Cu), cobalt (Co), nickel (Ni), germanium (Ge), indium (In), gallium (Ga), and silver [Ag (U.S. Geological Survey 1967)]. Iron (Fe), manganese (Mn), and barium (Ba) deposits have also been exploited, and mercury (Hg) is present in some areas of Missouri (Proctor *et al.* 1974). Pb-Zn mining was focused in three major areas of the state: The so-called "Old" and "New" Lead Belts, in southeastern and south-central Missouri; and the Tri-State Mining District of southwestern Missouri, which extends into eastern Kansas and Oklahoma (Figure 1). Of these areas, mining presently occurs only in the New Lead Belt.

Much of the historic Pb-Zn exploitation in Missouri utilized relatively inefficient (by today's standards) technology and preceded the advent of environmental concerns and regulations. Consequently, abandoned mines and unvegetated tailing deposits are present throughout the Old Lead Belt and Tri-State Mining District (e.g., Schmitt *et al.* 1984; Niethammer *et al.* 1985; Czarneski 1985, 1989; Neuberger *et al.* 1990). Contaminated surface and ground waters are particularly problematic in the Tri-State District, where the weathering of pyritic waste rock has acidified runoff and exacerbated the leaching of metals (e.g., Proctor *et al.* 1974). The carbonate-dominated geochemistry of the Old Lead Belt has generally precluded problems of this magnitude (e.g., Gale and Wixson 1986). In the New Lead Belt, environmental controls and more advanced bonification technologies (Wixson and Jennett 1975) have generally prevented the problems that characterize the inactive mining districts (Gale *et al.* 1973; Duchrow 1983; Czarneski 1985; Gale and Wixson 1986; Smith 1988); however, no statewide study has been conducted to determine the overall extent and magnitude of metals pollution from mining in Missouri streams.

The enzyme δ-aminolevulinic acid dehydratase (ALA-D) in the vertebrate erythrocyte catalyzes the formation of porphobilinogen (PBG), a hemoglobin (Hb) precursor, from aminolevulinic acid (Finelli 1977). ALA-D activity is highly sensitive to Pb; inhibition in humans typically occurs earlier and at lower blood-Pb concentrations than other symptoms of Pb poisoning (Secchi *et al.* 1974). Because ALA-D activity is also relatively easy to measure, inhibition of ALA-D activity has long been used as a biomarker of Pb exposure in humans (Granick *et al.* 1972; Secchi *et al.* 1974) and waterfowl (Dieter 1979).

The activity of ALA-D in fish blood is also depressed by Pb. Inhibition of erythrocyte ALA-D is specific for Pb; reduction in activity occurs rapidly and can be detected at exposure concentrations near the "no effect" level; and the enzyme system appears to be present in many fishes (Hodson *et al.* 1977). Schmitt *et al.* (1984) and Dwyer *et al.* (1988) reported that blood ALA-D activity was a useful biomarker of sublethal Pb exposure in fish from Missouri streams contaminated by Old Lead Belt tailings.

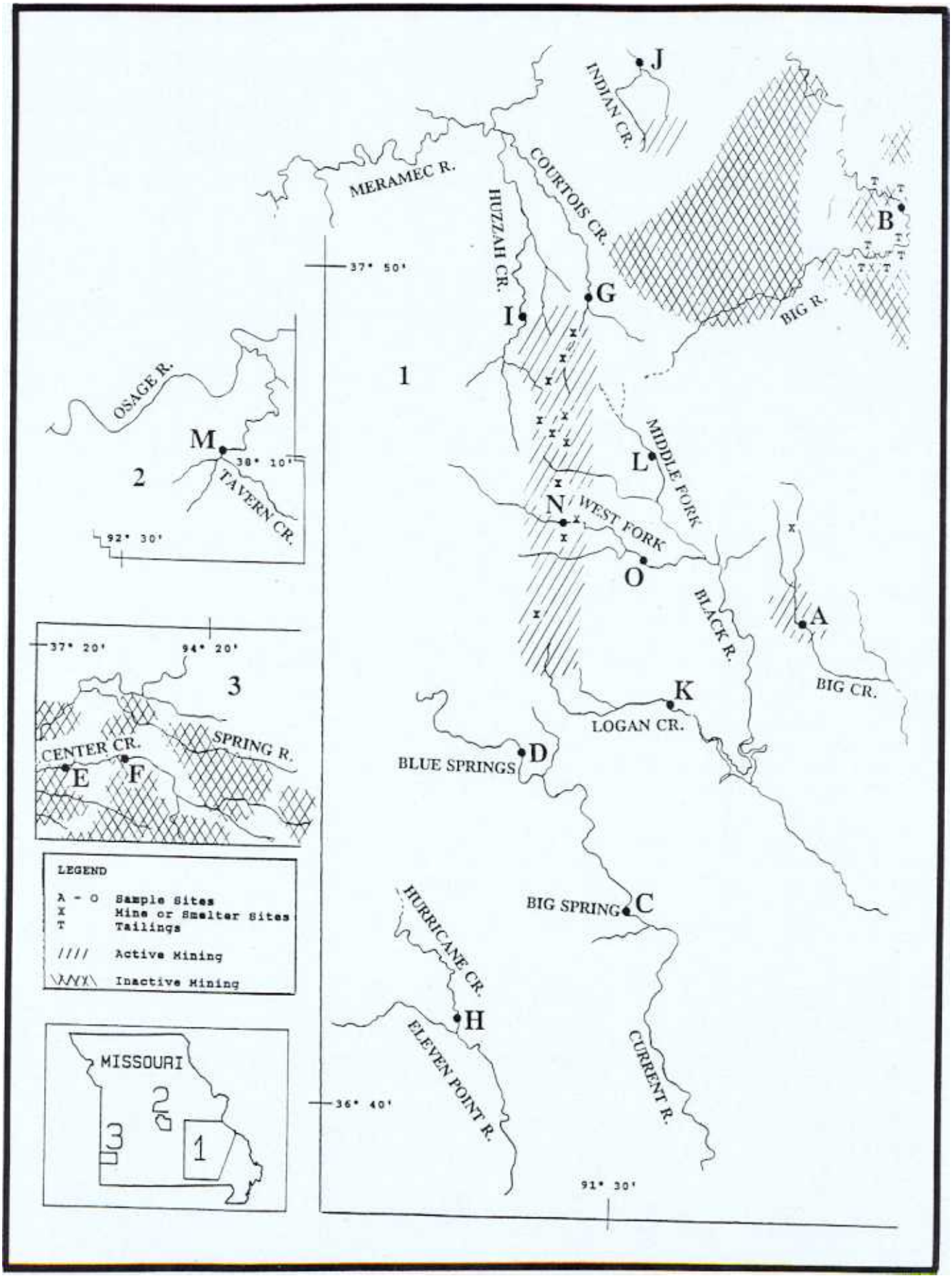


Figure 1. Location of sampling sites in relation to past and present mining operations.

Our main objective in this study was to verify and calibrate the biomarker of Pb exposure (ALA-D activity) for use in a statewide assessment of metals pollution from Pb-Zn mining, and to determine whether metals other than Pb and Zn affect ALA-D activity. Our overall approach was to collect suckers (Pisces: Catostomidae) from reference (no history of mining), positive control (documented historical pollution), and suspect (downstream from past or present mining activities, but not previously studied) sites in each of the three Missouri Pb-Zn mining districts. Previous studies (Schmitt *et al.* 1984; Dwyer *et al.* 1988) found that erythrocyte ALA-D in fishes was depressed by Pb, but that the response seemed to be ameliorated by Zn.

We therefore examined for a broad suite of potentially toxic elemental contaminants. ALA-D activity, and concentrations of metals and other elements in blood and carcasses were measured in individual fish from each site, and quantitative relations between the biomarker and elemental concentrations and other variables were determined. We also modified the field and laboratory procedures used to measure the biomarker.

Methods of Study

Study Area. Fish, water, and sediments were collected at eight suspect sites in the New Lead Belt and at two in the Tri-State Mining District (Table 1, Figure 1). In the New Lead Belt (Washington, Reynolds, and Iron Co.), the sites were situated as close as possible to mining or mine-related sources of metals to area streams. In Southwestern Missouri, sites on Center Creek upstream and downstream from an abandoned mining complex near Oronogo (Jasper Co.) were sampled; Zn has historically been elevated in Center Creek (Proctor *et al.* 1974).

In the Old Lead Belt, the Big River was sampled between BonTerre and Desloge (Washington Co.), downstream of the primary sources of tailings to the river. This site, which was also sampled in earlier studies (Schmitt *et al.* 1984, Dwyer *et al.* 1988), represented the positive control. Big Creek, which drains a small historic mining area near Annapolis, Mo. was included because Cd levels there are typically high (Proctor and Sinha 1978).

Big Creek also receives smelter discharges (Table 1). Reference sites included Hurricane Creek, a tributary of the Eleven Point River (a Designated Wild and Scenic River) situated within the Mark Twain National Forest in Shannon Co.; at the outlet of Big Spring, a conduit spring tributary of the Current River situated within the Ozark National Scenic Riverways in Carter Co.; and at Blue Spring, a state Natural Area that is also a tributary of the Current River in Shannon Co.

Because Big Spring and Hurricane Creek may be affected by Pb mining in the future (U.S Forest Service 1987), they were included to obtain baseline information. In 1975, Blue

Spring also showed evidence of slight metals enrichment during periods of high discharge (Barks 1978). An additional reference site (Tavern Creek, Cole Co.) was included to obtain information on more fish species, and because Tavern Creek supports a population of the Niangua darter (Etheostoma nianguae), a threatened species (Williams *et al.* 1989).

Field Procedures. At each site, a minimum of 10 suckers (Catostomidae) were sought, with the preferred species being black redhorse (Moxstoma duquesnii), golden redhorse (M. erythrurum), and northern hog sucker (Hypentelium nigricans). At Hurricane Creek, only white suckers (Catostomus commersoni) could be captured. At Tavern Creek, species from other families were collected along with several catostomids, and at Blue Spring, only sunfishes (Centrarchidae) could be obtained (for more detail, see Table 6 or 7 in results section).

Fish from all streams were captured by electrofishing in July, 1989 and held alive in ambient water until collection at the site had been completed. Electrofishing at Big Spring was conducted in January, 1990. Fish from Blue Spring were collected by hook and line in January, 1990. Immediately after collection, fish were subdued with a sharp blow to the head.

A blood sample (5 mL) was obtained by caudal peduncle puncture using a disposable 5-mL syringe equipped with a 21-gauge needle (Becton-Dickinson). To prevent clotting, the needle and syringe were rinsed with sodium heparin (1760 USP units/mL) immediately prior to blood collection. Freshly collected blood (1 mL) was dispensed into a 2-mL Cryovial® (Corning) and immediately frozen in liquid nitrogen (Hodson et al. 1977); this subsample, kept frozen at -80°C, was used for determinations of ALA-D activity and concentrations of Hb and total protein (TP).

The remainder of the blood sample was dispensed into a pre-numbered borosilicate glass culture tube (15 x 85 mm, Kimble), capped, and kept chilled on ice. The glass tubes and their polyethylene caps had been previously acid-cleaned and stored in polyethylene, as described by Schmitt *et al.* (1984). They were not heparinized. Upon return to the laboratory ($\leq$ 48 h), the subsamples in glass tubes, which were for blood metal determinations, were frozen (-25°C) until analysis.

After blood collection, the fish were measured (TL, mm) and weighed (g), and a scale sample was collected for age determination. A mid-ventral incision was made, through which the sex and gonadal state of the fish was determined, and a small (0.5 cm^3) piece of liver tissue was removed for another study. The remainder of the fish was double-bagged in polyethylene, chilled, transported to the laboratory, and frozen (-25°C) for subsequent determinations of carcass metals concentrations.

Samples of water and sediment were collected for trace metal analysis concurrently with fish sampling at each site. Water was pumped directly from the stream with a battery-powered,

backflushing filtration system (Geofilter®, GeoTech Inc., Boulder, CO) through a 0.45-μM polycarbonate filter (Nuclepore) into an acid-cleaned, 0.5-L polyethylene bottle. The sample was immediately acidified to pH-2 with sub-boiled 16-M HNO_3, and stored on ice or refrigerated ($\leq$ 4°C) until analyzed for trace metals ($\leq$ 72 h). A composite sediment sample was collected from a depositional zone at each site with an Eckman dredge. The sediment samples were chilled and stored in acid-cleaned 1-L glass jars, then frozen until analyzed for metals. No water samples were collected at Big Spring or Blue Spring; however, information on water and sediment metals is collected regularly by the U.S. National Park Service.

Laboratory Procedures. The blood samples stored at -80°C were thawed at room temperature immediately prior to analyses of Hb, TP, and ALA-D. Each of these analyses was performed in duplicate (i.e., two aliquots of each sample were analyzed).

The ALA-D procedure was modified from that used in earlier studies (Schmitt *et al.* 1984; Dwyer *et al.* 1988). One mL of cold, freshly prepared 0.1-M phosphate buffer, pH 6.5 (30.4 mL of 1-M Na_2HPO_4 + 69.7 mL of 1-M NaH_2PO_4 diluted to 1 L with ultrapure H_2O), was added to about 250 mg of whole blood in a glass culture tube and homogenized at 0°C with a Tissue Tearor® (Biospec Products, Bartlesville, OK). Of this mixture, 200 μL were placed in a 12- x 25-mm polyethylene centrifuge tube to which was added 300 μL of cold, freshly prepared aminolevulinic acid-Triton X (ALAT) solution (34 mg δ-aminolevulinic acid hydrochloride in 25 mL of 0.5% Triton X-100 in phosphate buffer, pH 6.5).

Each day, standards (0, 9.9, 19.9, 29.8, 39.8, and 49.7 nmol of PBG in ALAT, made up to 500 μL in ALAT) were prepared and carried through the procedure with the blood samples. The tubes containing 500 μL of samples and standards were vortexed and incubated in a water bath at 37°C for 1 h, after which the ALA-PBG reaction was stopped by adding 500 μL of TCA-$HgCl_2$ solution (4 g of trichloracetic acid and 2.7 g of $HgCl_2$ in 50 mL of ultrapure H_2O, stored in a dark glass bottle) to each sample and standard. Samples and standards were centrifuged for 5 min at 10,000 g, immediately after which 500 μL of each supernatant was pipetted into a glass culture tube (12 x 75 mm), and 500 μL of freshly prepared Ehrlich's-PAB solution [1 g of 4-dimethylaminobenzaldehyde (PAB) in 55 mL of Ehrlich's reagent (1.7 g of $HgCl_2$ in 30 mL of H_2O, 420 mL of 17.4-M HAc, and 100 mL of 70% $HClO_4$)] was added.

After incubating the mixture in the dark for 15 min at room temperature, the absorbance of the standards and samples was read at 553 nM with a spectrophotometer. Using the molecular weight of PBG (226.2) and the absorbance of the standards, a standard curve of the form $y = a + bx$, where y = absorbance and x = nMol PBG, was computed using linear regression for each group of samples. ALA-D activity in each sample was computed and expressed in units of nmol PBG/mL blood homogenate/h based upon its measured absorbance.

For TP determinations, 25 μL of the blood homogenate was mixed with an additional 1 mL of phosphate buffer and analyzed by a spectrophotometric dye-binding procedure (Bradford 1976; Bio-Rad Laboratories 1981) using bovine serum albumin as a standard. Results were reported in units of mg TP/mL of blood. For Hb, 100-μL aliquots of blood homogenate in phosphate buffer were analyzed by the cyanomethemoglobin spectrophotometric method (Larsen and Snieszko 1961; Sigma Chemical Co. 1984) using bovine Hb as a standard. Results were reported in units of mg Hb/mL of blood. Fish scales were examined in the laboratory with the aid of a microfiche reader. The number of completed annuli were estimated according to the general procedures given by Ambrose (1983).

At the laboratory where the analyses for metals and other elements were performed, fish carcass samples were ground in a meat grinder and weighed, then refrozen. Sediment samples were thawed, mixed, weighed, and refrozen. All frozen samples were freeze-dried (Labconco Freeze Dryer 8), then further homogenized in a mixer-mill (Spex Industries Model 8000). For blood samples, the moisture lost during freeze-drying was used to determine percent moisture. For fish carcasses and sediments, moisture content was determined by oven drying (Fisher Isotemp®) a separate sample aliquot to constant weight at 103-105°C.

For elemental analyses by inductively coupled plasma emission spectroscopy (ICP) and selenium (Se) analysis by atomic absorption (AA) spectroscopy with hydride generation, 0.5-g aliquots of dried sample or 50-mL aliquots of filtered water were digested with 15 mL of sub-boiled 16-M HNO_3 and 2.5 mL of concentrated sub-boiled $HClO_4$. The samples were refluxed overnight, then 2 mL of sub-boiled 12-M HCl were added. They were then heated gently, cooled, and diluted to 50 mL with ultrapure H_2O, and stored in polyethylene bottles.

The analytes of interest in blood, fish carcass, and water samples were preconcentrated for ICP in 30-g aliquots of the HNO_3-$HClO_4$ digestates by adding 1 mL of 1-g L^{-1} Indium and 1 mL of high-purity ammonium acetate buffer. The pH was adjusted to 6.5 with 1% NH_4OH [6.0 for samples high in Ca and P, to reduce the precipitation of $Ca_3(PO_4)_2$], and 1 mL of 10% Diethyldithiocarbamate was added. The mixtures were centrifuged at 20°C for 15 min at 15,000 rpm, the liquids were decanted, and 0.3 mL of high-purity 16-M HNO_3 was added to the precipitates. The samples were heated to 95°C in a water bath to dissolve the precipitates, diluted to 3 mL with ultrapure H_2O, and stored in polyethylene bottles.

Arsenic (As) concentrations were measured by hydride generation AA in separate 0.5-g aliquots of dried fish carcass, blood, and sediment samples, and in 50-mL aliquots of filtered water. These subsamples were digested with the HNO_3-$HClO_4$ procedure described previously, but without the final 2-mL addition of HCl. For Hg determinations by cold-vapor AA, separate 0.5-g aliquots of dried blood, fish carcass, and sediment samples and 10-mL aliquots of water were digested with 10 mL of sub-boiled 16-M HNO_3 and refluxed for 2 h. The samples were diluted to 50 mL with 1% (v/v) HCl and stored in flint glass bottles.

ICP analyses were performed with a Jarrel-Ash Model 1100, Mark III plasma emission spectrophotometer standardized with a series of seven standards containing 36 elements. The detection limit was determined by taking ten integrations of the zero standard; three times the standard deviation of the mean was used as the detection limit. Instrumental quality control samples were then analyzed to check the operation of the instrument. If the values were acceptable, the samples were then analyzed. Standards were run every 10-15 samples to check for drift. If the drift was more than 5%, the instrument was restandardized. The final detection limit for each element was further increased by 4% of the magnitude of the spectral interferences from the other elements.

Arsenic concentrations in fish carcass digestates were analyzed with a Perkin-Elmer (PE) AA spectrophotometer [either a Model 603 or 3030 (B)] equipped with a PE MSH-1 hydride generation accessory and electrodeless discharge lamp. Aliquots of blanks and samples were diluted to 10 mL with 4% (v/v) $HClO_4$, with the amount of sample varying with the As concentrations. For As in other sample matrices and all Se analyses, the PE AA spectrophotometers were equipped with Varian VGA-76 hydride generators. A PE-403 AA with a Technicon autosampler and pump was used for cold-vapor Hg analyses. The samples were reduced to Hg vapor with hydroxylamine and $SnCl_2$ prior to instrumental analyses.

Elemental concentration and detection limits in all matrices except water were reported by the analytical laboratory in dry-weight units. We converted concentrations in blood and carcass samples to wet-weight equivalents using each sample's moisture content.

Quality Assurance. Quality control for determinations of elemental concentrations by ICP and AA included evaluations of accuracy through analyses of reference materials (National Bureau of Standards bovine liver, and National Research Council of Canada dogfish), procedural blanks, and spiked samples; and of precision through duplicate sample analyses. All analyses of reference materials were within certified limits. Average recovery of spikes ranged from a low of 91% (mean, SD=6.1%) for boron (B) to 106% (SD=4.3%) for Cu. No blanks contained any element at concentrations above the instrumental detection limits.

Statistical Analyses. The Statistical Analysis System for personal computers (SAS Institute 1985) was used for all data handling and statistical analyses. A probability level of $P \leq 0.05$ was used in all tests of hypotheses. All data were analyzed in wet weight form to conform with the actual measurements of ALA-D activity. In accordance with previous studies (Schmitt *et al.* 1984; Dwyer *et al.* 1988), ALA-D, blood-Pb (Pb-B), and carcass-Pb (Pb-C) were also standardized to TP and Hb concentrations, and analyzed statistically in standardized form.

Heterogeneity of variance, which is assumed to be absent in most parametric tests of statistical significance, was examined by testing for the presence or absence of relations between cell means and standard deviations (Steele and Torrie 1980; Dwyer *et al.* 1988). These tests revealed that about half of the variables required log transformation (ln x) prior

to further statistical analysis (Table 2). Except for As-C and Cu-C, the transformations succeeded in eliminating relations between means and standard deviations. All results presented here are based on analyses of appropriately transformed values (Table 2).

Two-way analysis of variance (ANOVA) using partial sums of squares (Type III) and the Tukey-Kramer (T-K) method were used to compare and test differences among location and species means, and to test for interactions. Partial sums of squares allowed us to separately test for location and species differences after accounting for species or location effects, respectively, and location by species interaction effects. The T-K method is recommended for unbalanced designs and for controlling the potentially large experimentwise-error associated with making numerous comparisons of means (SAS Institute 1985). Thus, even though not all species were caught at all locations, the confounding of location effects with species effects were minimized, and the reliability of the minimum significant differences (MSDs) presented is high. Relations among variables were investigated further with simple correlation, analysis of covariance, and stepwise multiple regression.

Detection Limits. Concentrations of one or more metals were below detection limits in every observation. Because the detection limit was computed independently for each element in each observation, the limits (e.g., censoring levels) are presented as approximate ranges for those variables that were censored (Table 3). For variables not listed, all values were above detection limits. Thallium in blood and carcass and beryllium in blood were not used in any statistical analyses because, of the 156 observations, 148 or more measurements were below detection limits.

We examined the distributions of Pb detection limits for individual observations ($n = 107$) to determine if information would be lost if these censored values were eliminated from the analyses. There were no discernible relations between ALA-D and Pb-B detection limits (Figure 2). Results presented henceforth are based only on values above detection limits.

Results

Sediment. Sediments from Courtois Creek, Huzzah Creek, Indian Creek, Tavern Creek, and the West Fork of the Black River contained only sand and gravel, and were consequently not analyzed for metals. Concentrations of Pb, Zn, and Cd were elevated relative to the reference site at the two Center Creek sites, the Big River, and Big Creek (Table 4). Pb concentrations were highest in Big River sediments, whereas Cd and Zn were highest in sediments from the downstream Center Creek site. Center Creek sediments (both sites) also contained elevated Cr and Al concentrations. Pb and Zn concentrations were also higher in sediments from the Black River (both sites) than at other reference or suspect sites, and Middle Fork sediments contained the highest Ni concentrations (Table 4). Concentrations of As were noticeably lower in sediments from the two springs than elsewhere (Table 4).

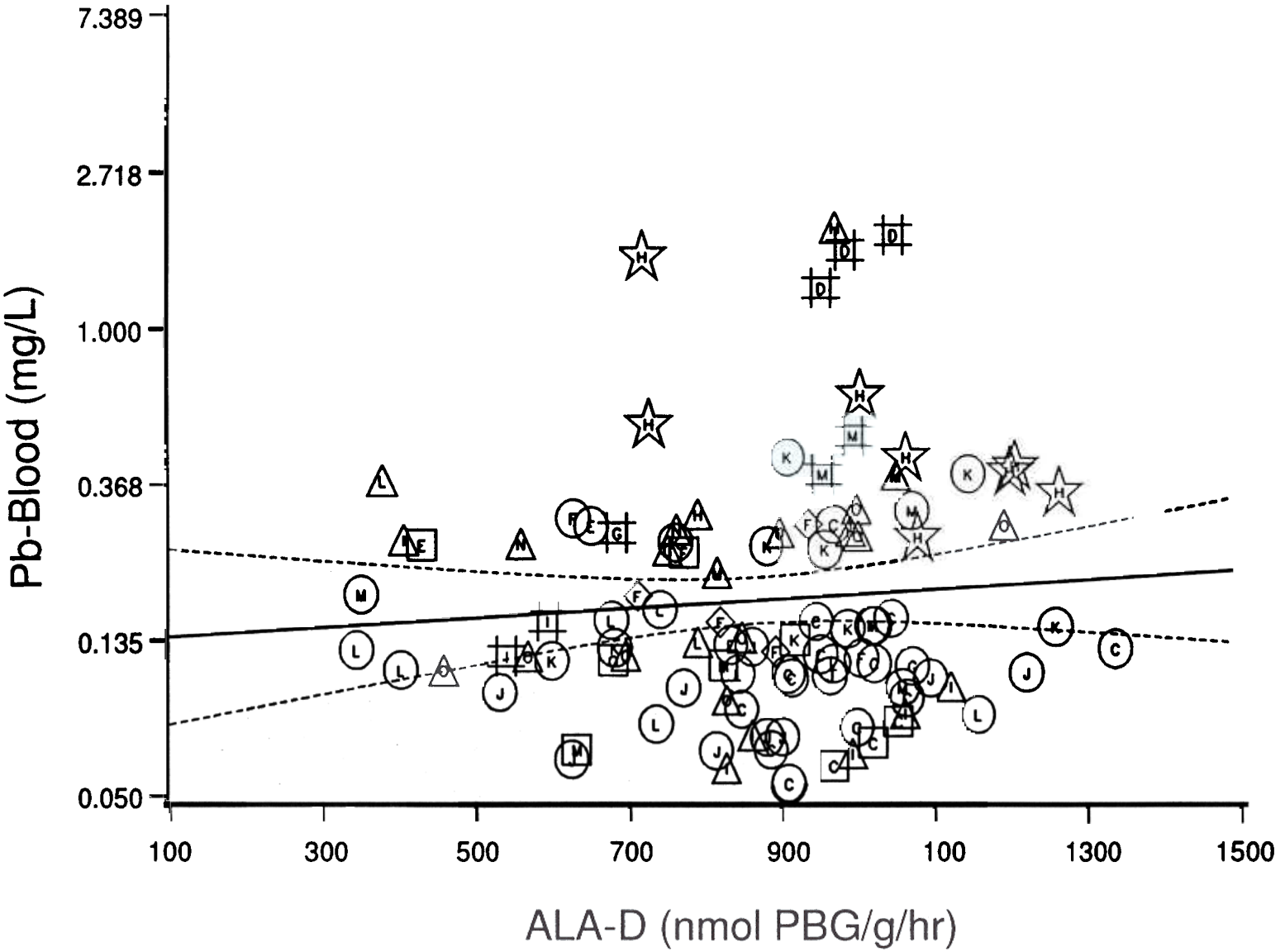


Figure 2. Ln-linear regression between Pb concentration in fish blood (y-axis) and ALA-D activity (x-axis) for those values which did not exceed detection limits. Letters represent the sample site from which the fish were collected (Figure 1, Table 1). Circle, black redhorse; square, golden redhorse; triangle, hog sucker; diamond, shorthead redhorse; star, white sucker; hash, green sunfish, longear sunfish, rock bass, and yellow bullhead. The dashed lines are the 95% confidence interval for the regression line (solid line): ln(Pb-B) = -2.0114 + 0.000311*ALA-D, n=104, R^2=0.01, P>0.38.

Water. Concentrations of many metals in water were below detection limits, and no sample was collected from either Big or Blue Spring (Table 5). Pb was only detected in water from Big River, and as expected, Zn in water was very high (0.26 mg/L) at the downstream Center Creek site. Cd was detected at low concentrations in water at only four sites, one of which (Tavern Creek) was a reference site (Table 5). Cu in water was slightly elevated at Logan Creek, whereas Fe in water was elevated at the downstream Center Creek site, and Mn was highest at the three sites affected by historic mining activities (Big River and Center Creek) and at the upper west fork of the Black River site where there are ongoing mining activities (Table 5). Concentrations of the other metals in water varied among sites, but were generally low.

Whole Fish and Blood Variables. Except for Mn-B, Cd-C, and Hg-C, none of the measured variables showed a significant location by species interaction (Tables 6 and 7), which indicates that inter-species differences are consistent from site to site (i.e., a measured variable was higher or lower in a given species regardless of the site from which it came). ALA-D activity and TP, but not Hb, differed significantly among locations (Table 6). As expected, unadjusted ALA-D, ALA-D adjusted for Hb (ALA-D/Hb), and ALA-D adjusted for TP (ALA-D/TP) were lowest in fish from the Big River, the positive control. ALA-D and ALA-D/Hb were highest at Hurricane Creek, one of the reference sites, and ALA-D/TP was highest at Big Spring, another reference site, ALA-D activity was generally highest at reference sites and lowest at Big Creek and at the other sites affected by historic mining activity. TP was highest at the middle fork of the Black River and lowest at Big Spring. Of these five blood measurements associated with ALA-D activity, two also differed significantly among species; ALA-D/Hb and ALA-D/TP were highest in white suckers and lowest in yellow bullheads.

Pb, Zn, and Cd concentrations differed significantly among locations in both blood (Table 6) and carcasses (Table 7). Of these metals Pb-B, Pb-C and Cd-B did not differ significantly among species. As expected, both Pb-B and Pb-C were highest at Big River. These metals were lowest at Huzzah Creek in blood and at Big Spring and Blue Spring in carcass (Tables 6 and 7). Zn-B and Zn-C were highest at the downstream Center Creek site and lowest at Hurricane Creek (Zn-B) and Logan Creek (Zn-C) (Tables 6 and 7). Cd-B and Cd-C were highest at Big Creek and lowest at Big Spring (Cd-B) and at Tavern Creek and Logan Creek (Cd-C). Among species, Cd-B was highest in hog suckers and lowest in green sunfish. Zn was highest for shorthead redhorse in both blood and carcass, lowest for white sucker in blood, and lowest in yellow bullhead for carcass (Tables 6 and 7).

Of the other metals measured, only Al and Mn concentrations differed significantly among locations in both blood and carcass; Mn-B and Mn-C also differed among species (Tables 6 and 7). Al-B was lowest at Big River, where Al-C was highest. Al-B was highest at Blue Spring and Al-C was lowest at Big Spring. Mn-B was highest at the upper west fork of the Black River and lowest for Big Spring, whereas Mn-C was highest at Big River and lowest at Blue Spring.

Mn-B was highest in hog suckers and lowest for black redhorse, golden redhorse, and green sunfish. Mn-C was highest in shorthead redhorse and lowest in rock bass. Cr and Ni differed significantly among locations only in blood, with Cr-B highest at Courtois Creek and lowest at Huzzah Creek (Table 6).

Ni-B was highest at the middle fork of the Black River and lowest at Indian Creek. Cu, Fe, Hg, and Se differed significantly among locations only in carcass; As-C, Hg-C, and Se-C also differed significantly among species (Table 7). Copper was highest at Blue Spring and lowest at Tavern Creek and the downstream Center Creek site. Iron was highest at Indian Creek and lowest at Blue Spring.

Mercury was highest at Tavern Creek and lowest at Big River and both Center Creek stations. Selenium was highest at Big Creek and lowest at Big River. As-C was highest in hog suckers and lowest in black redhorse, golden redhorse, white suckers, and yellow bullheads. Hg-C was highest in green sunfish and lowest in shorthead redhorse. Se-C was highest in green sunfish and lowest in yellow bullheads.

Of the more general variables measured, wt, TL, and age differed significantly among locations; wt and TL also differed significantly among species (Table 7). These variables associated with fish size were obviously confounded. The heaviest, longest, and one of the two oldest groups of fish came from Big Spring (Big River fish were the same age); 25 of these 28 fish were black redhorse. The lightest and shortest fish came from Blue Spring, which were all centrarchids. The youngest fish came from the west fork of the Black River. In general, the heaviest and longest fish were golden redhorse, the lightest were green sunfish, and the shortest were longear sunfish.

Lastly, correlation analysis revealed significant relations (i.e., $P<0.05$) between blood and carcass values for five metals. Of these, four of the five correlations were positive: (Cd, $r=0.664$; Mn, $r=0.269$; Pb, $r=0.815$; and Zn, $r=0.409$); and one was negative (Al, $r=-0.262$).

ALA-D in Relation to Other Variables. Three of the ALA-D adjustment variables (blood- and carcass-moisture and TP) differed significantly among locations (Tables 6 and 7). In contrast, Hb did not differ significantly among locations. Carcass-moisture also differed significantly among species (Table 7). Before continuing with the analyses, we determined that among-location and -species differences in ALA-D activities were not confounded by differences in the adjustment variables. When the location and species means of adjustment variables differed significantly, this was accomplished by examining correlations with unadjusted ALA-D. The results of these tests revealed no significant correlations ($P>0.18$).

Once the confounding factors had been eliminated, we performed separate stepwise regression analyses of unadjusted ALA-D--one for blood-metals and one for carcass-metals. These analyses were done separately because there were only half as many Pb-B as Pb-C

values above detection limits. Because stepwise regression analysis only uses observations in which a value is present for all variables (SAS Institute 1985), only a small fraction of the original data set would have been used. Also, by separating the two data sets, we were able to compare the effectiveness of blood and carcass information at describing relations between ALA-D and metal concentrations.

To use as much of the information as possible, a stepwise analysis was first done with the full compliment of blood or carcass variables. Next, the one variable with the smallest set of measured (i.e. above detection limit) values and which was not a significant variable in the model was removed from the set of possible variables. The stepwise analysis was then repeated with the reduced-rank data set. Each removal of a variable from the set of all possible variables increased the number of observations used in the analysis. This process was repeated until 90% or more of the available Pb-B or Pb-C observations were used.

The stepwise regression analyses showed that Pb was consistently the most important factor in both the blood- and carcass-metals models. For blood-metals, Pb-B alone accounted for 52-62% of the variation in ALA-D activity (i.e., $\underline{R}^2=0.62$, $\underline{n}=15$--after Cr-B removed; $\underline{R}^2=0.60$, $\underline{n}=25$--after Ni-B removed; $\underline{R}^2=0.52$, $\underline{n}=43$--after Cd-B). For the carcass-metals model, Pb-C alone accounted for 43-51% of the variation in ALA-D activity (i.e., $\underline{R}^2=0.43$ $\underline{n}=60$--after Be-C removed; $\underline{R}^2=0.44$, $\underline{n}=69$--after As-C removed; $\underline{R}^2=0.51$, $\underline{n}=75$--after Se-C and Hg-C removed; $\underline{R}^2=0.49$, $\underline{n}=84$--after Ni-C removed). The final models for ALA-D activity based on both blood- and -carcass metals data included Pb, Zn, and TP (Table 8).

Neither species nor sex were significant with these variables added to the final stepwise models (Table 8). Throughout the variable-step stepwise analyses, the only changes from the significant variables in the final blood-metals model occurred at $\underline{n}=15$, when Mg-B was added, and at $\underline{n}=25$, when Zn-B was removed. The only change from the significant variables that made up the final carcass-metals model occurred at $\underline{n}=75$, when TP was replaced by Hb and TL was added. Within the blood and carcass data sets, the only significant correlation to occur among Cd, Pb, and Zn was between Zn-B and Cd-B ($\underline{R}=-0.492$, $\underline{n}=26$). Collectively, these findings suggest that erythrocyte ALA-D responds to Pb-B and, to a lesser extent Zn-B, as reported in earlier studies (Schmitt *et al.* 1984, Dwyer *et al.* 1988), and that other metals have no discernible effect on ALA-D activity under the conditions of our study.

Estimation of Pb Concentrations from ALA-D. To determine whether Pb-B and Pb-C could be estimated without knowledge of any metal concentrations, we modeled Pb-B and Pb-C as functions of the more easily measured non-metal variables--species, sex, wt, TL, age, ALA-D (linear and quadratic), Hb, and TP. Weight, ALA-D, and TP accounted for 66% of the variance in Pb-B (Table 9). Similarly, species, wt, ALA-D, Hb, and TP accounted for 69% of the variance in Pb-C (Table 9). ALA-D alone accounted for 52% of Pb-B and for Pb-C 48% (Figures 3 and 4). In the Pb-B model, hog suckers and black redhorse constituted

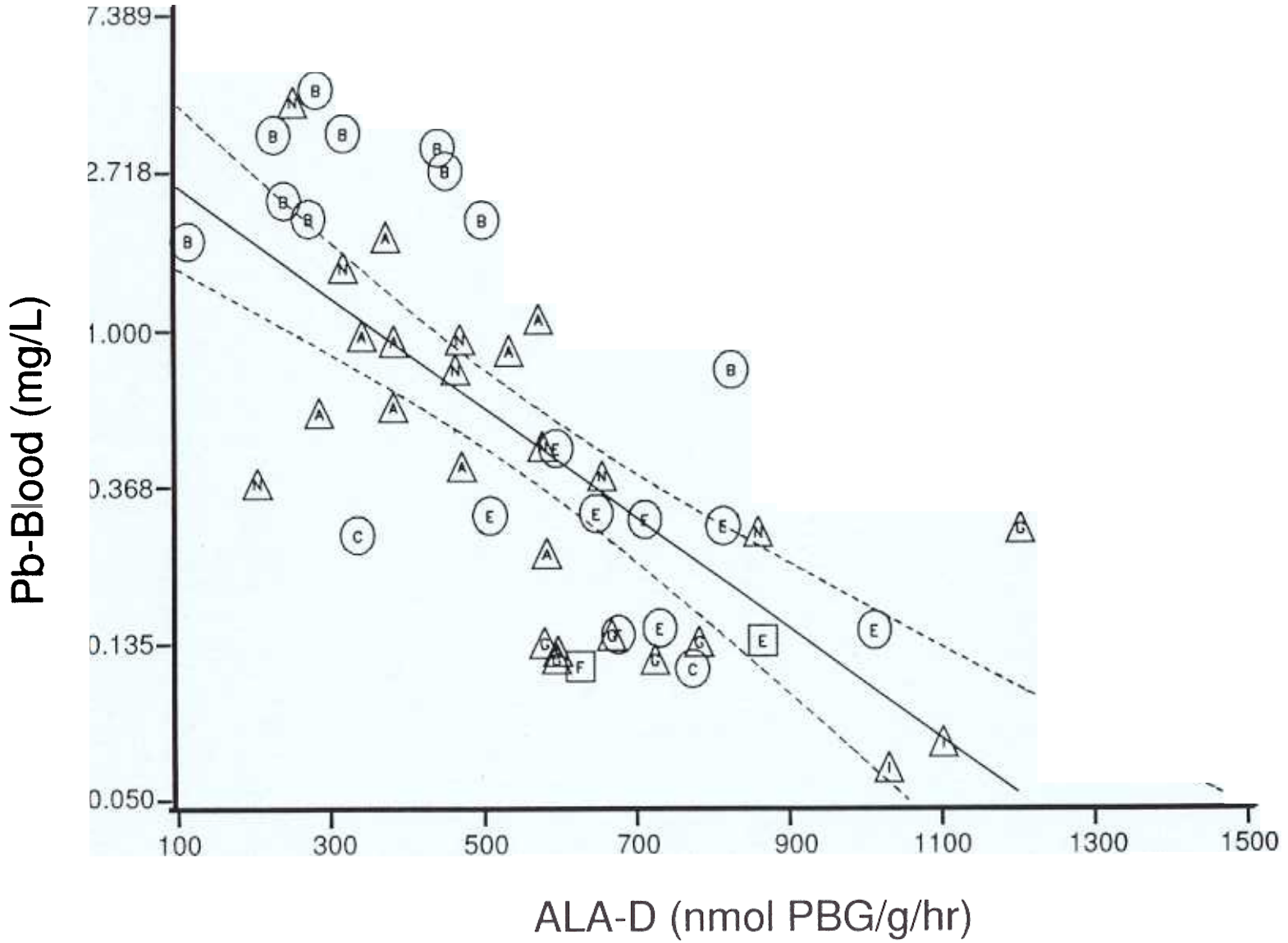


Figure 3. Ln-linear regression between Pb concentration in fish blood (y-axis) and ALA-D activity (x-axis) for measured values that exceeded detection limits. Letters represent the sample site from which the fish were collected (Figure 1, Table 1). Circle, black redhorse; square, golden redhorse; triangle, hog sucker. The dashed lines are the 95% confidence interval for the regression line (solid line): $\ln(\text{Pb-B}) = 1.2644 - 0.00352 * \text{ALA-D}$, $n=48$, $R^2=0.52$, $P=0.0001$.

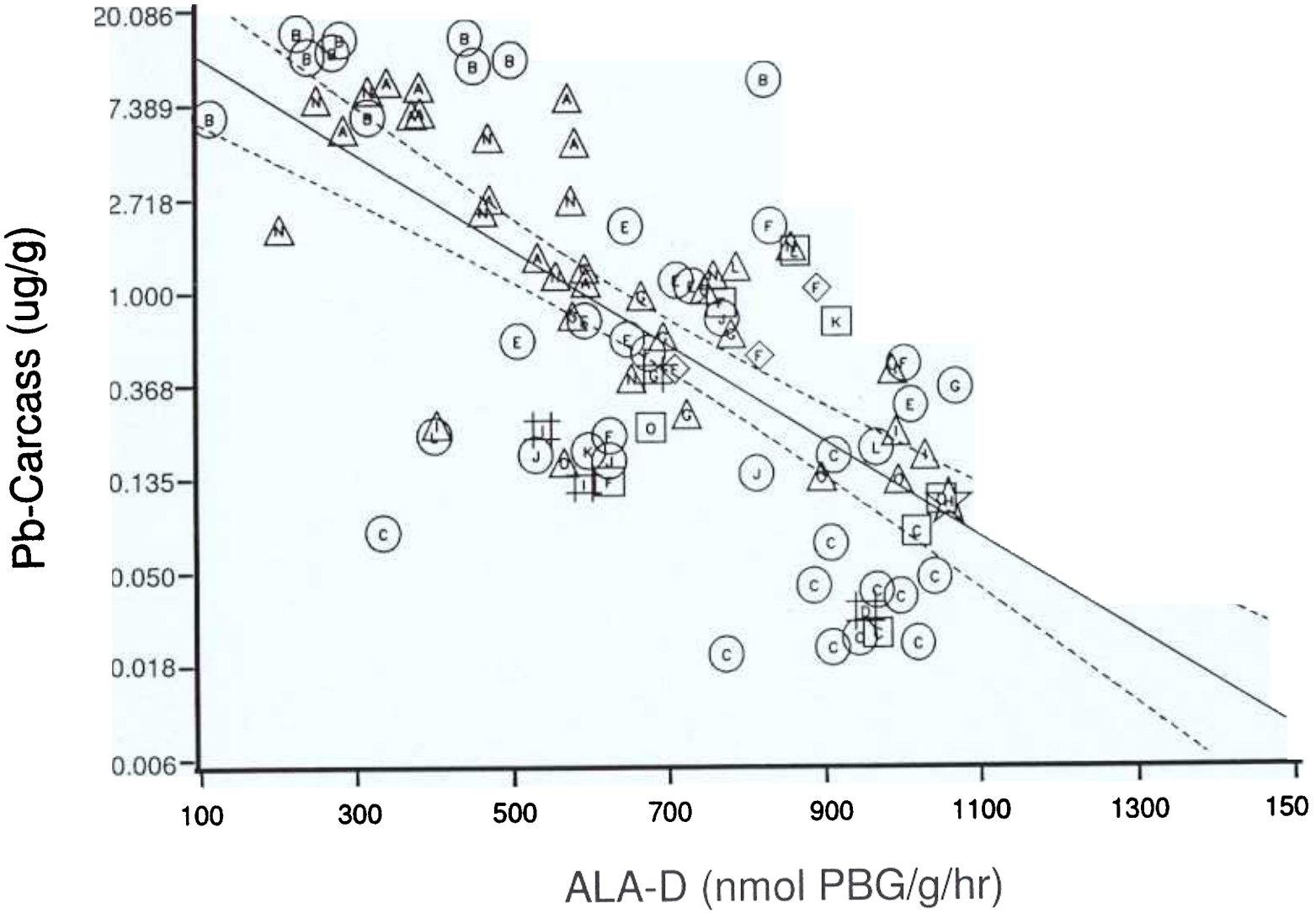


Figure 4. Ln-linear regression between Pb concentration in fish carcass (y-axis) and ALA-D activity (x-axis) for measured values that exceeded detection limits. Letters represent the sample site from which the fish were collected (Figure 1, Table 1). Circle, black redhorse; square, golden redhorse; triangle, hog sucker; diamond, shorthead redhorse; star, white sucker; hash, green sunfish, and yellow bullhead. The dashed lines are the 95% confidence interval for the regression line (solid line): ln(Pb-B) = 3.0264 - 0.00513*ALA-D, n=91, R^2=0.48, P=0.0001.

all but two of the observations (the other two were golden redhorse) because of the loss of data at the detection limits; thus, it was improbable that species would have been a significant factor in this model. The lack of a location by species interaction in the ANOVAs for any of the variable used in these models (Tables 6, 7, and 9) supports the hypothesis of a constant slope for these models, with among-species differences in the mean concentration of Pb (i.e., different intercepts).

Inspection of the relation between Pb-B and Pb-C revealed that, although these variables were correlated, the relation between them was quadratic rather than linear (Figure 5). Moreover, the models for Pb-B and Pb-C adjusted for TP or Hb (Table 10) produced results similar to those for unadjusted Pb (Table 9); however, none described the variation in Pb-B or Pb-C better than the unadjusted model. Moreover, log-log and log-linear relations among ratios are prone to induced correlations (Jackson *et al.* 1990). And lastly, the only significant correlations with sediment- or water-metals were carcass- with sediment-lead ($\underline{r}=0.83$, $\underline{n}=8$, $\underline{P}<0.012$) and water- with sediment-nickel ($\underline{r}=1.0$, $\underline{n}=3$, $\underline{P}<0.032$). There were insufficient measurable lead levels in the water for correlation analysis.

Discussion

Metals in Missouri Streams. For the most part, the results of our study were as we expected; concentrations of Pb, Cd, and Zn were highest in all media, and ALA-D activity was lowest, at the sites affected by historic mining activity in Old Lead Belt (Big R.) and Tri-State Mining District (Center Creek), with greater evidence of contamination in Center Creek site downstream of Oronogo then upstream (Tables 6 and 7, Figs. 3 and 4). Conversely, metals concentrations were generally lowest and ALA-D activity highest at the reference sites. Most of the New Lead Belt sites were intermediate, but a gradient was evident, with Pb-B, Pb-C, and ALA-D activity in fish from the upper West Fork of the Black River approaching those from the upstream Center Creek site.

In fish from Big Creek, Cd-B and Cd-C were 3- to 10-fold higher than at the other sites affected by historic mining wastes; Pb-B was elevated; and ALA-D activity was depressed by ~50% relative to the reference sites (Tables 6 and 7 and Figs 3 and 4). Relative to concentrations in sediments, Cd-B and Cd-C were much higher in fish from Big Creek than from the other sites affected by historic mining (cf. Tables 4, 6, and 7) suggesting that the Cd in Big Creek was more bioavailable. This stream has a well documented history of ecological effects stemming from smelter discharges (Ryck 1974) and elevated metals from an abandoned mine near Annapolis, Mo. (Proctor and Sinha 1978).

ALA-D as a Biomarker of Pb Exposure. The general form of the relation between ALA-D and Pb-B is remarkably similar across taxa. Studies with fishes (e.g., Hodson *et al.* 1977; Schmitt *et al.* 1984; Dwyer *et al.* 1988; this study) waterfowl (Dieter 1979; Dieter and Finley 1978; Blus *et al.* 1991), raptorial birds (Henny *et al.* 1991), humans (Granick *et al.*

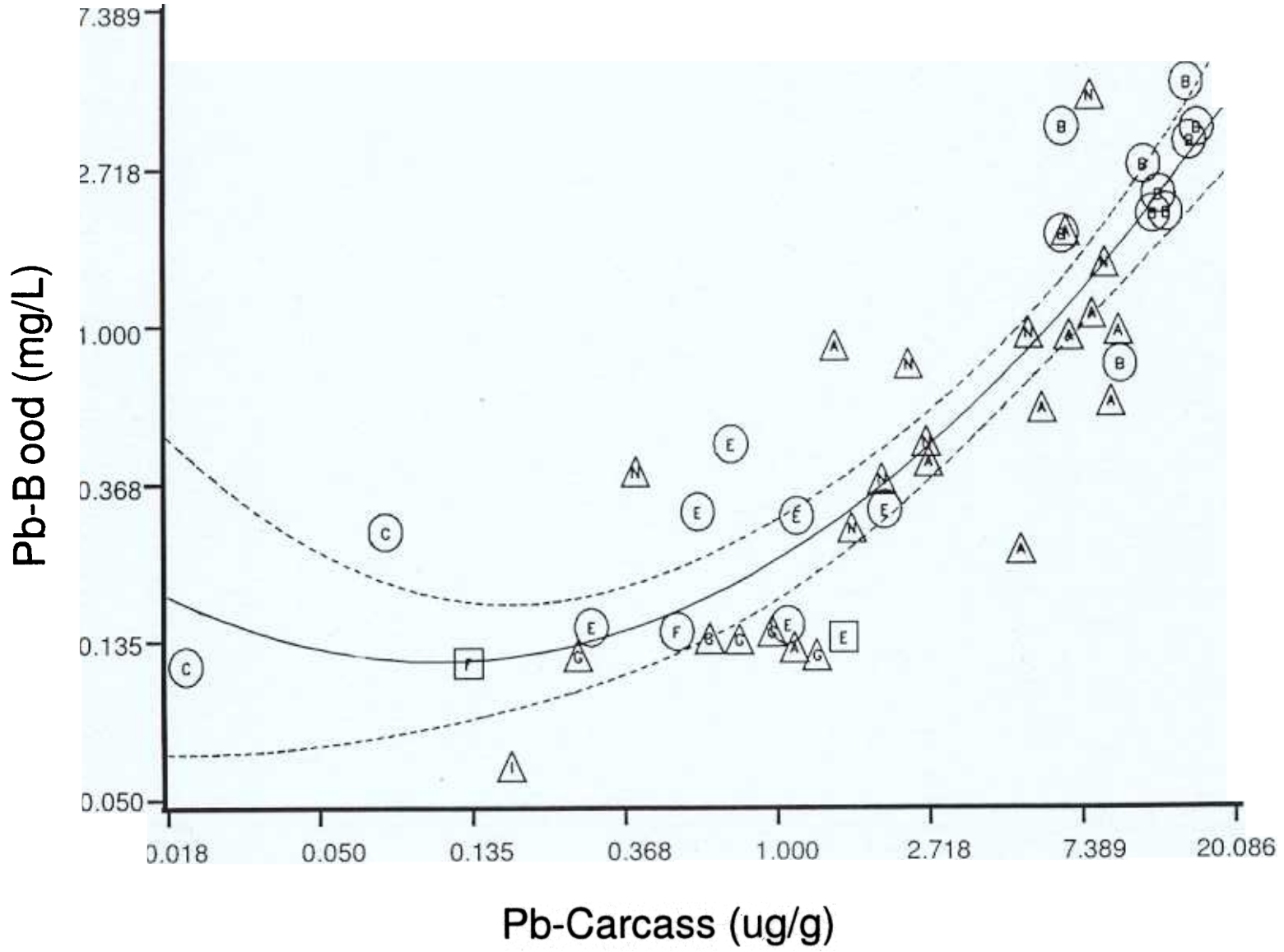


Figure 5. Ln-ln regression between Pb concentration in blood (y-axis) and Pb concentration in fish carcass (x-axis) for measured values that exceeded detection limits. Letters represent the sample site from which the fish were collected (Figure 1, Table 1). Circle, black redhorse; square, golden redhorse; and triangle, hog sucker. The dashed lines are the 95% confidence interval for the regression line: $\ln(\text{Pb-B}) = -1.450 + 0.5595*\ln(\text{Pb-C}) + 0.1339*(\ln(\text{Pb-C}))^2$, $n=48$, $R^2=0.77$, $P=0.0001$.

1972), and other mammals (e.g., Wigfield *et al.* 1986) have generally revealed curvilinear (i.e., log-linear) negative relations extending down to Pb-B levels of 0.2-0.3 mg/L (2-3 μg/dL). Pb-B generally accounts for about 50% of the variation in field-study ALA-D activity, and more (70%) in laboratory studies.

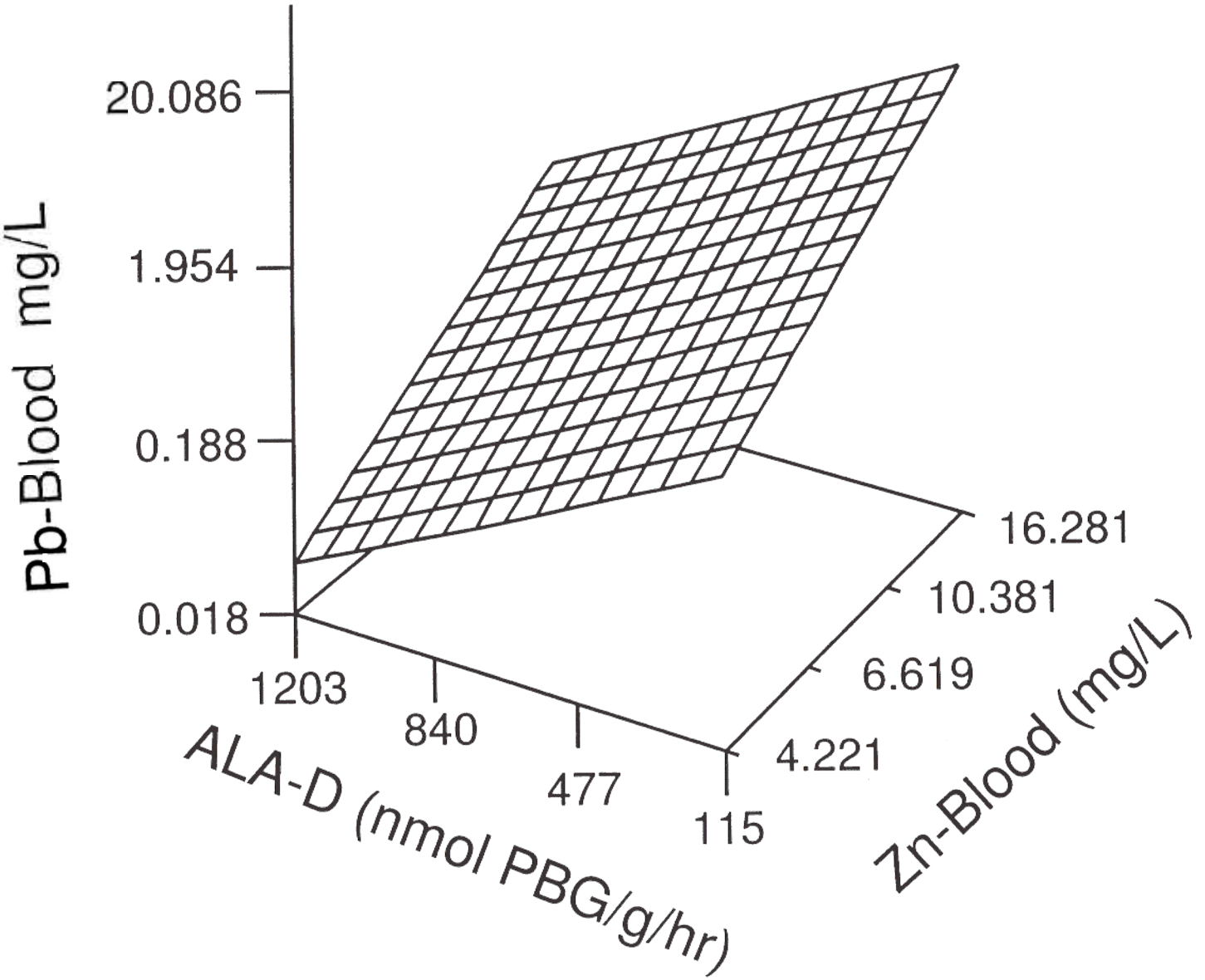
Several factors have been proposed to account for the fact that although erythrocyte ALA-D inactivation is specific for Pb-B, as much as half of the variability in ALA-D remains unexplained by Pb-B. Wigfield *et al.* (1986) attributed variability in laboratory rats and other mammals to a pronounced Pb-dependent shift in the pH optimum for the ALA-D assay. Such an effect has not been demonstrated in fishes, however (Hodson *et al.* 1977; Krajnovic-Ozretic and Ozretic 1980). In our study (Table 8) and in previous field studies conducted in Missouri (Schmitt *et al.* 1984; Dwyer *et al.* 1988), where other metals co-occur with Pb, Zn-B also accounts for a statistically significant portion of the variability in ALA-D activity.

The effect of Zn-B on ALA-D in these studies has consistently been positive (i.e., positive regression coefficients suggestive of an ameliorative effect of Zn on Pb-induced ALA-D inactivation), which is consistent with the well known ability of Zn to reactivate ALA-D in vitro (e.g., Finelli 1977; Krajnovic-Ozretic and Ozretic 1980; Wigfield *et al.* 1986), and the ability of waterborne Zn to increase ALA-D activity in laboratory studies (Hodson *et al.* 1977).

One objective of our study was to determine, through the selection of sites with diverse contaminant sources and pollutant metal ratios, the extent to which metals other than Pb and Zn influenced ALA-D activity in environmentally exposed fishes. In vivo laboratory studies with fishes have generally shown that ALA-D is much less sensitive to other metals than to Pb, whereas many inactivate the enzyme in vitro (Hodson *et al.* 1984). Hodson *et al.* (1984) interpreted this finding as indicating that metals other than Pb and Zn do not reach the erythrocyte in vivo.

In Daphnia magna, ALA-D activity was lowered by either Pb or Cd alone, but combinations of Pb with either Cd or Zn reduce the inhibitory effect of Pb (Berglind 1986). In our study, environmentally derived metals other than Pb and Zn (notably Cd) were present at elevated concentrations in fish blood from contaminated sites, but they had no discernable effect on ALA-D activity. Relative to reference sites, Cd-B was elevated 2-3 fold at Center Creek and 10-fold at Big Creek (Table 6), yet Pb-B and Zn-B were the only blood metals included in regression models for ALA-D activity (Table 8). No metals other than Pb-C or Zn-C were included in models based on carcass concentrations either (Table 8).

Over the range of concentrations for which we have Pb-B and Zn-B data, the slope of the Pb-B vs. ALA-D response is similar (Figure 6). The relative flatness of the response surface reflects the additive, rather than interactive, effect of Zn-B on the relation. Hence, we conclude that at the concentrations and under the conditions of our study (e.g., hard water),



Fi[illegible] -In-[illegible] idealized plot among Pb -concentration ood (z- concentratio and LA- (x-axis) for measured values that exceeded etection mits

metals other than Pb and Zn do not appear to influence ALA-D activity, and that the ALA-D method may underestimate Pb-B by as much as 4- to 5- fold in the presence of high Zn-B (Figure 6).

The caveats stated in the preceding paragraph derive at least in part from analytical detection limits. We selected ICAP for determining elemental concentrations because it provided for a greater breadth of analyses (i.e., more elements) than AA at substantially lower costs, albeit at the expense of sensitivity for some elements of interest. We reasoned that the sensitivity of the ALA-D assay to Pb-B in catostomids had already been documented (Schmitt *et al.* 1984), and ICAP would enable testing of the effects of other elements on the response while still permitting quantitation of potentially harmful Pb-B (i.e., that which would measurably inhibit ALA-D). This strategy was only partly successful; although there was enough data to enable exploratory statistical analyses and development of models for Pb-B and Pb-C as functions of ALA-D activity and other readily measured variables, the loss of information at the detection threshold precluded examination of the effects of some metals at low concentrations. In retrospect, AA analyses of fewer samples for fewer elements would have been advantageous.

In human medicine, 1-1.5 mg/L (10-15 μg/dL) Pb-B is considered cause for concern, with levels of 4 mg/L associated with high risk of injury involving numerous body systems (Mushak *et al.* 1989). Avian wildlife is considered Pb-poisoned at Pb-B concentrations of 0.2-0.6 mg/L, varying with species (Feierbend and Myers 1984; Anderson and Havera 1985). For example, in tundra swans (Cygnus columbianus) contaminated by Pb from mine tailings in Idaho, Pb-B of 1-10 mg/L apparently caused moribundity and death (Blus *et al.* 1991).

ALA-D was depressed by about 75% in these most severely affected swans, and they were anemic. Anemia is also common in Pb-poisoned raptorial birds displaying reduced ALA-D activity (Hoffman *et al.* 1981, 1985). Some fishes, however, may be more tolerant of Pb-B than birds or mammals.

Previous Missouri field studies (Schmitt *et al.* 1984; Dwyer *et al.* 1988) reported Pb-B >5 mg/L and ALA-D inhibition of 65%, and no evidence of anemia or neurotoxicity. Haux *et al.* (1986) reported ALA-D inhibition of 88% in whitefish (Coregonus spp.) exposed to Pb from mining without apparent hematological effect, although some effects on plasma ionic regulation and glucose metabolism were suggested. In contrast, neurotoxicity (i.e., black tail syndrome) in laboratory-exposed rainbow trout (Oncorhynchus mykiss) is associated with ALA-D inhibition $\geq 50\%$ (Hodson *et al.* 1984), and there was evidence of impaired bone strength and biochemical dysfunction in longear sunfish from contaminated reaches of the Big River where ALA-D inhibition was about 60% (Dwyer et al. 1988).

Thus, fishes may vary in their sensitivity to Pb-B and ALA-D inhibition; although some of this variability may reflect adaptation or acclimation to high Pb levels. Moreover, the

studies necessary to determine the significance of chronic exposure of fishes to Pb have not been conducted. Although "harmful" exposure of fishes to environmental Pb remains undefined, the utility of ALA-D as a biomarker of exposure and bioavailability does not.

Through this and previous studies, ALA-D has been shown to be sensitive to environmental Pb, and it seems is relatively unaffected by metals other than Zn at concentrations likely to occur in the environment. Moreover, the observed damping of the ALA-D response to Pb by Zn also implies that estimates of Pb-B based upon ALA-D activity will be biased downward in the presence of elevated Zn-B, rendering estimates conservative. As noted earlier, the slopes of the Pb-B vs. ALA-D relations appear similar across taxa; however, the intercepts vary, which suggests that although the enzyme system is present in all taxa, sensitivity varies somewhat. Consequently, a calibration exercise resembling this study should be undertaken before the biomarker is applied elsewhere.

The precision of the ALA-D assay, as evidenced by the confidence limits displayed in Figure 3, are sufficient for estimating mean Pb-B and Pb-C in fish collected as a group from a suspect site, and consequently, for determining whether the site has been contaminated. As performed, however, the confidence limits associated with estimated Pb-B and Pb-C in individual specimens are so wide as to preclude the classification of all but the most heavily contaminated individuals as such. In human medicine (Granick *et al.* 1973) and in laboratory rats (Wigfield *et al.* 1986), inter-individual variation in ALA-D activity can be reduced, and the precision of estimated Pb-B correspondingly increased, by expressing measured ALA-D activity as a proportion of "reactivateable" ALA-D activity. This approach, which has not been evaluated in fish, may also reduce apparent differences among taxa, and could also eliminate the need for the measurement of collateral variables such as TP, Hb, or fish size. It will probably not eliminate the aforementioned bias associated with Zn, however.

Recommendations

From this and previous Missouri field studies (Schmitt *et al.* 1984, Dwyer *et al.* 1988), it is apparent that ALA-D activity is an effective method for assessing Pb exposure of groups of fishes from Missouri streams. In Missouri streams, where Pb is present, often Zn is also present. The presence of Zn and its ameliorative effect on ALA-D deactivation by Pb suggests that ALA-D activity is a conservative estimate of Pb exposure of Missouri stream fishes. Although often present in high concentrations in the same Missouri streams where Pb and Zn are found, metals other than Pb and Zn do not appear to influence ALA-D activity. Thus, we recommend measuring ALA-D activity over directly measuring Pb concentrations in fish blood in order to estimate Pb exposure of Missouri stream fishes, since it is a more rapid, less-costly, and simpler alternative.

Acknowledgements

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